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The CreC regulator of *Escherichia coli*, a new target for metabolic manipulations

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Abstract

The CreBC (carbon source responsive) two-component regulation system of *Escherichia coli* affects a number of functions, including intermediary carbon catabolism. The impact of different *creC* mutations (a Δ*creC* mutant and a mutant carrying the constitutive *creC510* allele) on bacterial physiology was analyzed in glucose cultures under three oxygen availability conditions. Differences in the amounts of extracellular metabolites produced were observed in the null mutant compared to the wild-type strain and the mutant carrying *creC510*, and shown to be affected by oxygen availability. The Δ*creC* strain secreted more formate, succinate, and acetate, but less lactate in low aeration. These metabolic changes were associated to differences in AckA and LdhA activities, both of which were affected by CreC. Measurement of the NAD(P)H/NAD(P)$^+$ ratios showed that the *creC510* strain had a more reduced intracellular redox state, while the opposite was observed for the Δ*creC* mutant, particularly at intermediate oxygen availability conditions, indicating that CreC affects redox balance. The null mutant formed more succinate than the wild-type strain in both low aeration and no aeration. Over-expression of the genes encoding phosphoenolpyruvate carboxylase from *E. coli* and a NADH-forming formate dehydrogenase from *Candida boidinii* in the Δ*creC* mutant further increased the yield of succinate on glucose. Interestingly, the elimination of *ackA* and *adhE* did not improve significantly the production of succinate. The diverse metabolic effects of this regulator on the central biochemical network of *E. coli* make it a good candidate for metabolic engineering manipulations to enhance the formation of bioproducts such as succinate.

Introduction

The survival of an organism depends, at least in part, on its ability to sense and respond to changes in the environment. In bacteria, global regulators control the transcription of genes in response to specific external stimuli and metabolic signals, finely tuning different aspects of their physiology to overcome environmental challenges. In *Escherichia coli*, seven global regulators (ArcA, Crp, Fis, Fnr, Ihf, Lrp, and NarL) directly modulate the expression of about one-half of all genes (1). This facultative aerobe is able to adapt its metabolism to different oxygen availability conditions through the concerted action of a network of regulators, including the global regulators ArcAB and Fnr (2-4). These regulators affect many metabolic pathways, allowing the cells to reach an adequate redox balance in any given condition. There is a very close association between carbon and electron flows, and even small differences in oxygen availability have been observed to elicit profound effects in the distribution of carbon fluxes (5).

CreBC (for *carbon source responsive*) is a global sensing and regulation system affecting genes involved in a variety of functions, including enzymes of the intermediary catabolism (6). Previous studies have shown that the *creABCD* operon is activated during growth in minimal media when (i) glycolytic carbon sources are being fermented or (ii) during aerobic growth when low-molecular-weight fermentation products are used as carbon sources (6). While *creB* and *creC* encode the two component system (i.e., a cytoplasmic response regulator and a membrane-associated sensor kinase, respectively), *creA* is a hypothetical open reading frame and *creD* encodes an inner-membrane protein of unknown function (7). CreC, originally designated PhoM, was first described as a phosphate donor for the PhoB protein, a response regulator that controls the expression of the *pho* regulon. This regulon includes genes involved in cytoplasmic inorganic phosphate homeostasis, such as *phoA*, encoding an alkaline phosphatase, and is controlled by PhoBR (8, 9). PhoR autophosphorylates when the concentration of inorganic phosphate falls below a critical threshold. In *phoR* null mutants, activation of the *pho* regulon depends on CreC (10).

77 The genes that are known to be under the control of CreBC (i.e., the *cre* regulon) are (i) the
78 *ackA/pta* operon, the products of which catalyze the conversion of acetyl-coenzyme A (CoA)
79 into acetate and ATP, (ii) *talA*, that encodes one of the two transaldolases of the pentose
80 phosphate pathway, (iii) *radC*, that encodes a RecG-like DNA recombination/repair function,
81 (iv) *malE*, the first gene in the *malEFG* maltose transporter operon, (v) *trgB*, that encodes an
82 ADP-ribose pyrophosphorylase; and (vi) three other genes (*creD*, *cbrA*, and *cbrB*), potentially
83 related to resistance to colicin and other antimicrobials, that have not yet been assigned a specific
84 function (6, 7, 11). A direct repeat consensus DNA sequence, termed *cre* tag (5'-
85 TCAC*nnnnnn*TTCAC-3', where *n* represents any nucleotide), was defined based on the analysis
86 of the region upstream from the genes known to form the *cre* regulon, and observed to be
87 required for the control of gene expression *in vivo* (7). Genome-wide expression profiling with
88 DNA microarrays has revealed that CreBC also affects the expression of *cbrC*, responsible for
89 colicine E2 resistance; *mokB*, an overlapping regulatory peptide which enables *hokB* expression;
90 and the uncharacterized genes *mppA*, *ynaI*, *yafU*, and *yafE* (12).
91
92 The capability of global regulators to affect multiple metabolic pathways makes them useful
93 tools for metabolic engineering, as they can be used to change the flow of carbon and reducing
94 power simultaneously. This strategy has been used to manipulate bacterial metabolism to
95 enhance the synthesis of different bioproducts, specially reduced metabolites (13). On the other
96 hand, concern about the costs of energy used for aeration in bioprocesses has renewed attention
97 on the regulation of aerobic and anaerobic bacterial metabolism, as a means to achieve the
98 sustainable synthesis of a variety of bioproducts under these conditions (14). Among the
99 different regulatory systems of *E. coli*, ArcAB has attracted significant attention in the last years,
100 as manipulations in this sensor/regulator pair offer the possibility of directing carbon flow
101 towards the synthesis of reduced bioproducts in low aeration conditions (15-17). In our
102 laboratory we have analyzed the effect of *arcA* mutants on the central carbon catabolism of *E.*
103 *coli* using glucose and gycerol in microaerobiosis and anaerobiosis, and observed that mutations
104 in *arcA* resulted in significant increases in the synthesis of polyhydroxyalkanoates (18-21) and
105 ethanol (22, 23). In these studies, the constitutive allele *creC510* was observed to affect the

intracellular redox state, enhancing carbon catabolism in an *arcA* genetic background so that part of the excess reducing power generated by the *arcA* mutants was consumed by the augmented amount of carbon intermediates due to *creC510*, further increasing the synthesis of reduced products (18).

The results obtained with the *creC510 arcA* double mutants suggested that the CreBC system could also be an interesting target for metabolic manipulations in *E. coli*, and prompted us to further investigate its metabolic effects. The aim of this work was to evaluate the potential of CreC as a new tool for the design of bacterial strains suitable for the synthesis of different bioproducts. For this purpose, we characterized the effects of this regulator on central carbon metabolism, analyzing physiological traits, carbon flow, and redox balance, focusing on low oxygen availability conditions in the presence of excess carbon source. Succinate was selected as a model metabolite, and several metabolic manipulations were implemented in a Δ*creC* mutant to evaluate the potential of this genetic background for the synthesis of this carboxylic acid.

Materials and methods

Bacterial strains, mutant construction, plasmids and oligonucleotides. All *E. coli* strains were derivatives of K1060, a K-12 strain, and are listed in Table 1, along with the plasmids used in this study. All null mutants reported in this work were constructed by allelic replacement (24). Briefly, a kanamycin resistance cassette (FRT-*kan*-FRT) was amplified by PCR from template plasmid pKD4 with the corresponding primers (Table 1). The purified PCR product was electroporated into *E. coli* K1060 carrying pKD46 (a helper plasmid that expresses the λ-Red functions). Insertion of the FRT-*kan*-FRT cassette into the correct locus was confirmed by colony PCR of kanamycin resistant recombinants. For the construction of mutant K1060C, bearing the constitutive *creC510* allele, a previous step was needed. *E. coli* strain 58-161, F-,SR (*creC510*) carrying pKD46 was transformed with the FRT-*kan*-FRT cassette, in order to add a selection marker near the *creABCD* operon in the intergenic region limited by the convergent open reading frames *yjjX* and *trpR*, located 2.5 kb upstream from *creABCD*. The resulting

mutant, *E. coli* 58KF, was used as the donor strain in P1 transduction, in which K1060 was the recipient strain. Kanamycin resistant clones were selected and the presence of the *creC510* allele was confirmed by PCR and DNA sequencing. The *kan* cassette was removed in all the strains by transforming them with the thermosensitive plasmid pCP20 (25), encoding the *Saccharomyces cerevisiae* FLP recombinase. The excision of *kan*, as well as gene deletion, was confirmed by PCR. Plasmid pCP20 was removed by two consecutives passages at 42ºC. Inactivation of *ackA* in *E. coli* CEA1060 (Δ*creC* Δ*adhE* Δ*ackA*) was further corroborated by determining the amount of acetate present in the supernatant of cultures grown in M9 minimal medium supplemented with glucose at 30 g liter^{-1} as described below (the CEA1060 mutant produced 50% less acetate than its parental strain CE1060).

Growth media and culture conditions. The medium used for shaken-flask experiments was M9 minimal medium containing (per liter of deionized H_2O): 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, 1.0 g of NH_4Cl, 0.4 g of $MgSO_4$, 0.01 g of $CaCl_2$, and 0.06 g of ammonium iron(III) citrate. $MgSO_4$, $CaCl_2$, and ammonium iron(III) citrate were added to the medium after autoclaving and cooling. Glucose was used at 30 g liter^{-1} as the sole carbon source in all experiments. The aeration level was adjusted by the combination of rotational agitation and by the relation between the volume of culture medium (V_m) and the volume of the flask (V_f) as follows: for high aeration, 250 rpm and $V_m/V_f = 1/10$; for low aeration, 125 rpm and $V_m/V_f = ½$. For high aeration 250 ml erlenmeyer flasks were used, while 10 or 50 ml cylindric bottles (penicillin bottles) with cotton plugs were used for low aeration. Non-aerated cultures were implemented in sealed tubes filled with culture medium (10 ml) and agitated at 4 rpm to keep cells in suspension. Working cultures were inoculated from overnight pre-cultures (started from single colonies grown overnight on LB agar), in the same culture media and growth conditions to be used in the experiment (i.e., with the same V_m/V_f ratio). Non-aerated pre-cultures were inoculated with a low aerated pre-preculture to ensure adaptation of the cells to this condition. Isopropil-β-D-1-thiogalactopyranoside (IPTG, 0.1 or 1 mM), $NaHCO_3$ (100 mM) and antibiotics (kanamycin and ampicillin at 50 and 100 μg ml^{-1}, respectively) were added whenever needed.

Analytical determinations. The cell dry weight (CDW) was determined in cell pellets of 10-ml culture samples that were centrifuged for 10 min at 4°C and 10,000×*g*. The supernatant was separately stored for metabolite analysis. The cells were washed twice with the same volume of 150 mM NaCl, and finally dried in an oven at 65°C until constant weight. The supernatant was filtered through a 0.22-µm pore-size syringe filter (Chrom Tech Inc., Apple Valley, MN) and stored at 4°C for high-pressure liquid chromatography analysis (LC-20AT Prominance; Shimadzu Corp., Kyoto, Japan), equipped with an Aminex column HPX-87-H (Cat no. 125-0140; Bio Rad Laboratories Inc., Hercules, CA) at 50ºC. An UV detector (SPD-20AV; Shimadzu Corp.) set to 215 nm was used for the quantification of organic acids. The mobile phase consisted of 5 mM H_2SO_4, run at a flow rate of 0.6 ml min^{-1}. Peaks were identified by their characteristic retention times against a set of standards of known organic acids (Sigma-Aldrich Co., St. Louis, MO). Ethanol concentration was measured by using an enzymatic kit based on alcohol dehydrogenase (Sigma-Aldrich Co.). Glucose was measured in supernatants with the glucose oxidase-peroxidase method utilizing a commercial kit (Wiener Laboratorios SAIC, Rosario, Argentina).

The $NADH/NAD^+$ and $NADPH/NADP^+$ ratios were obtained from the content of each nucleotide, quantified in the pellet fraction of 1-ml culture samples. Samples were transferred to pre-cooled plastic tubes and the metabolic activity was quenched by immersion of the tubes in liquid N_2. Thawed samples were treated with 300 µl of either 0.2 M HCl [NAD(P)H extraction] or 0.2 M NaOH [$NAD(P)^+$ extraction]. Acid/alkaline extraction was carried out at 50ºC for 10 min, and samples were rapidly placed on ice to cool them at 0ºC afterwards. Suspensions were neutralized by dropwise addition of 1 M HCl or NaOH, and cellular debris was removed by centrifuging at 14,000×*g* for 5 min. Supernatants were then transferred to new tubes and immediately used for cofactor measurements. Nucleotide determination was performed as described by Bernofsky and Swan (26), using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as the final electron acceptor, as modified by Nikel et al. (22, 27, 28). The dinucleotide content was normalized to the CDW as indicated by Nikel and Chavarría (29). All reagents were purchased from Sigma-Aldrich Co.

193 ***In vitro* enzyme activity measurements.** The acetate kinase (AckA) assay is based on the
194 formation of acetyl-hydroxamate (30, 31). The assay mixture (1 ml final volume) consisted of 50
195 mM Tris·HCl (pH = 7.4), 10 mM $MgCl_2$, 10 mM ATP, 800 mM CH_3CO_2K, and 700 mM of
196 freshly neutralized NH_2OH. Working solutions of neutralized NH_2OH were freshly prepared by
197 dropwise addition of 3.5 M KOH to an equal volume of 4.0 M NH_2OH·HCl. The cell-free
198 extract was added to the reaction mixture, and incubated for 5 min at room temperature, after
199 which 1 ml of 10% (w/v) trichloroacetic acid was added, immediately followed by 1 ml of
200 freshly-prepared 1.25% (w/v) $FeCl_3$ in 1 N HCl. After another 5-min incubation period at room
201 temperature, the mixture was centrifuged at maximal velocity during 1 min, and the absorbance
202 the supernatant was read of at 540 nm. For D-lactate dehydrogenase (LdhA), the activity was
203 assayed by measuring the pyruvate-dependent reduction of NADH (32). The assay mixture
204 consisted of 50 mM sodium phosphate buffer (pH = 7.5), 25 mM pyruvate, and 7.5 mM NADH.
205 The cell-free extract was added to the reaction mixture to initiate the assay, and the rate of
206 change in the absorption at 340 nm was recorded at 30ºC using a microtiter plate reader.
207 Calibration curves were performed using lithium-potassium acetyl-phosphate and NADH for
208 AckA and LdhA, respectively. One unit of AckA or LdhA activity was defined as the quantity of
209 enzyme that catalyzes the formation of 1 µmol product in 1 min at 30°C. All reagents were
210 purchased from Sigma-Aldrich Co.
211
212 **Statistical analysis.** The reported experiments were independently repeated at least twice (as
213 indicated in the corresponding figure legend or table), and the mean value of the corresponding
214 parameter ± standard deviation is presented. The statistical significance between multiple
215 comparisons was obtained by a two-tailed Student's *t* test. Data were considered statistically
216 significant when $P < 0.05$.
217

Results

CreC affects growth and the fermentation profile of *E. coli* under low oxygen availability conditions. Previous reports suggested that the CreC dependent regulation is affected by aeration. To analyze this effect further, an *E. coli* strain carrying the wild-type *creC* (K1060), a *creC* deletion derivative (DC1060), and another carrying the constitutive *creC510* allele (K1060C) (Table 1), were grown in three levels of aeration (high, low, and no aeration). Growth and production of different metabolites were determined to characterize the metabolic responses of each strain to oxygen availability in M9 minimal medium cultures supplemented with 30 g liter^{-1} glucose.

Clear differences were observed in growth and in metabolite secretion, mainly between *E. coli* DC1060 and the other two strains, both of which showed a similar behavior in the three culture conditions (Table 2). Biomass formation at 24 h was similar in high and low aeration for all strains, but surprisingly strain DC1060 grew twice as much as the other strains with no aeration (see Fig. S1 in the Supplemental material). Metabolite distribution in highly aerated cultures was similar for all strains, with acetate as the main secreted product. In low aeration, *E. coli* K1060 and K1060C showed similar trends, while strain DC1060 secreted more formate (2-fold), succinate (13-fold), and acetate (1.5-fold), but approximately half the lactate than the other strains ($P < 0.05$). Differences in succinate production in non-aerated cultures were even more marked, as the null mutant produced 36% more of this metabolite than in low aeration, while the other strains (K1060 and K1060C) had very low accumulation levels (Table 2). Succinate formation in non-aerated cultures of the Δ*creC* strain was around 50-fold higher than in cultures of *E. coli* K1060 grown in these conditions (9.4 *versus* 0.2 mmol g_{CDW}^{-1}). Interestingly, and in contrast to what was observed in low aeration, in 24 h non-aerated cultures the null mutant accumulated more lactate (+60%), and less acetate (-60%) than the other two strains, while all strains accumulated similar amounts of formate (Table 2). While all strains produced higher amounts of ethanol when no aeration was supplied than in the other growth conditions, K1060C

246 was the strain that accumulated the highest levels, followed by wild-type *E. coli* K1060, and
247 strain DC1060 had the lowest ethanol formation.
248
249 **The effect of CreC over the fermentation profile and enzyme activities is mediated by its**
250 **partner response regulator CreB.** As CreC is the sensor protein of a two-component signal
251 transduction system (the partner response regulator is encoded by *creB*), the possibility that the
252 changes observed could be due to cross talk with other regulators was considered. To investigate
253 this possibility, the fermentation profile of a Δ*creB* derivative of strain K1060 (*E. coli* DB1060)
254 was analyzed in low aeration and no aeration, conditions in which the most significant
255 differences between *E. coli* DC1060 and the parental strain had been observed. The fermentation
256 profile was also analyzed in a double Δ*creB* Δ*creC* mutant (*E. coli* DBC1060).
257
258 While in low aeration the final biomass was the same for all the strains, 24 h non-aerated cultures
259 of the three null mutants produced 2-fold more biomass than the wild-type strain in this
260 condition (Table 2). When comparing the specific production of organic acids in both low
261 aeration and no aeration, all the deletion mutant strains produced comparable amounts of all
262 metabolites, with similar differences when compared to the wild-type strain. The same metabolic
263 profile was obtained in strains in which either component or both were inactivated, clearly
264 indicating that the effects observed are due to the regulation exerted by the two component
265 system CreBC.
266
267 **CreC influences the NADH/NAD$^+$ ratio.** When no aeration was supplied, ethanol levels were
268 lower in cultures of the Δ*creC* strain, while the *creC510* mutant accumulated the highest
269 amounts. These results suggested that CreC affects the redox state of the cells, as the formation
270 of this metabolite is associated to a high availability of reducing equivalents. To have an accurate
271 measurement of the intracellular redox state of each strain, the levels of cofactors
272 NAD(P)H/NAD(P)$^+$ were quantified *in vitro*, and their ratios determined.
273

While in high aeration the NADPH/NADP$^+$ ratios were higher than the NADH/NAD$^+$ ratios for all strains, the opposite was observed in non-aerated cultures. The ratios of both types of cofactors were similar in low aeration, except for the null mutant, in which this relationship was approximately the same in high and low aeration. Interestingly, the NADPH/NADP$^+$ ratios were observed to vary on a much wider range under the conditions tested, while variations in NADH/NAD$^+$ were more discreet. However, when the values obtained for each of the strains were compared, no significant variations were detected among the NADPH/NADP$^+$ ratios, while differences in the NADH/NAD$^+$ ratio were observed in the three aeration conditions. In all cases, the highest NADH/NAD$^+$ ratio was observed for the mutant carrying the constitutive allele *creC510* (*E. coli* K1060C), and in low aeration the deletion mutant (*E. coli* DC1060) had the lowest redox ratio values (Fig. 1). In high aeration, a condition in which CreC is believed to be inactive, the NADH/NAD$^+$ ratios differed slightly, and the highest value was observed for *E. coli* K1060C that has the constitutively active regulator. In low aeration, in which CreC is expected to be active, the differences were more conspicuous (Fig. 1). In this condition *E. coli* DC1060 showed clear differences with both the wild-type strain and the *creC510* mutant, with a NADH/NAD$^+$ ratio 47% lower than the value for *E. coli* K1060, and 66% lower than that of *E. coli* K1060C. These results indicate that, under oxygen limitation, CreC affects the availability of redox cofactors, promoting a more reduced intracellular environment. When no aeration was supplied, the wild-type strain and the null mutant behaved similarly, but strain K1060C showed a significantly higher NADH/NAD$^+$ ratio compared to the two other *E. coli* strains ($P < 0.05$).

AckA and LdhA activities are subjected to CreC regulation. The metabolic profiles of the strains showed differences in metabolite distribution, affecting several organic acids, mainly succinate, formate, lactate, and acetate. CreC has been reported to activate the *pta-ackA* operon (7), so the increase in acetate observed in the absence of CreC led us to investigate whether the variations on acetate synthesis could be attributed to differences in AckA (acetate kinase) activity. On the other hand, variations in the amount of lactate could be attributed to metabolic regulation or to differences in LdhA (lactate dehydrogenase) activity. However, LdhA is not considered to be regulated by CreBC (at least at the transcriptional level) since the *ldhA*

promoter region does not present a *cre* tag. In an attempt to elucidate these interrogants, strains K1060 and DC1060 were grown in the three aeration conditions indicated previously, and the specific activity of AckA and LdhA were determined *in vitro* during exponential growth (Fig. 2). In high oxygen availability, no significant differences were observed in the specific AckA or LdhA activities between both strains, while in low aeration the enzyme activities reflected the metabolic profile shown in Table 2. That is, the Δ*creC* strain had a higher AckA activity and a lower LdhA activity compared to the wild-type strain, in agreement with the higher acetate and lower lactate production in this condition. The specific activities of AckA and LdhA in the Δ*creB* mutants were also analyzed in low aeration to compare them with the corresponding metabolic profiles and, as expected, both strains behaved identically (data not shown). From these experiments it can be concluded that either directly or indirectly, CreC affects the activities of both AckA and LdhA.

In non-aerated cultures, however, there was no clear correspondence between enzyme activity and metabolite levels. Although *E. coli* DC1060 produced more lactate and less acetate than the parental strain in 24 h cultures, the LdhA and AckA activities (measured during the exponential phase) in the non-aerated cultures were approximately 50% lower and 200% higher, respectively ($P < 0.05$). The discrepancy observed in non-aerated cultures led us to make additional measurements in order to consider possible variations due to culture age: (i) metabolite levels were determined in the exponential cultures used for the initial enzyme determinations, and (ii) enzyme activities were also measured at the onset of the stationary phase of growth. Similar amounts of acetate were detected in the supernatants of exponential non-aerated cultures of both strains (15.6 mM in K1060 *versus* 12.6 mM in DC1060), but K1060 produced more lactate than DC1060 (0.7 mM *versus* undetectable levels, respectively) in this condition, in accordance with the differences observed in LdhA activity.

It must be noted that, in the absence of aeration, *E. coli* DC1060 produced more biomass and grew faster than the wild-type strain, making it difficult to choose the best moment along the growth curve for comparisons. For this reason, the cultures used for enzyme determinations were

harvested in what could be considered as exponential and early stationary phase in each case (sampling times are shown in Fig. S1 in the Supplemental material).

Although in early stationary non-aerated cultures the AckA activity did not match the observed trend in acetate formation, differences between these two parameters were more moderate than in the exponential phase (Fig. 2A). In contrast, the results obtained for LdhA in the early stationary phase of non-aerated cultures were quite different to those obtained in the exponential phase. The early stationary phase cultures of strain DC1060 displayed a higher LdhA activity compared to the wild-type K1060 (Fig. 2B), and produced more lactate (6.3 mM in DC1060 *versus* undetectable levels in K1060), in accordance with the larger amounts of lactate measured in 24 h non-aerated cultures (Table 2).

To further investigate this we measured pH in the cultures, as this parameter is known to affect LdhA activity (33). In high and low aeration the pH was observed to decrease throughout the exponential phase for all strains, and to remain stable upon entry into the stationary phase. No differences in pH evolution were observed among the strains except in the un-aerated cultures. In this condition, the wild type (K1060) grew very little, and the pH decreased only slightly, while the null mutant (DC1060) grew more, and had a much greater drop in pH (Fig. S1 in the Supplemental material).

Given that (i) the Δ*creC* mutant exhibited lower LdhA activity and lactate levels than the wild-type strain in all conditions except in stationary non-aerated cultures and (ii) appreciable differences in pH were only observed in this condition, it is apparent that the low pH could be at least partially responsible for the increase in LdhA activity observed in DC1060, that in turn correlates with higher lactate levels.

Manipulation of CreC as a genetic tool for the improvement of succinate formation. One of the most interesting properties of the *creC* null mutant was the augmented production of succinate, since this organic acid is a biotechnologically interesting compound (34). Many strategies have been performed so far to optimize the production of this metabolite (35), such as

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over-expressing carboxylating enzymes (36-39) and supplying carbon dioxide by the addition of sodium bicarbonate to the culture medium (39-41). Since the *creC* mutant produced higher amounts of succinate compared to the parental strain, additional strategies were tested to increase the titer of this metabolite in *E. coli* DC1060. Two plasmids were introduced in the strains: plasmid pEcPpc, carrying the carboxylating enzyme phosphoenolpyruvate carboxylase (Ppc^{Ec}) from *E. coli* (42), and plasmid pSBF2, carrying a NAD^+-dependent formate dehydrogenase from the methylotrophic yeast *Candida boidinii* ($FDH1^{Cb}$) (43-45). In both plasmids, the corresponding genes are under the control of the *lac* promoter, and their expression can be induced by IPTG. Taking into account that the Δ*creC* mutant produces high amounts of formate in low aeration, expression of $FDH1^{Cb}$ could help convert the excess formate to CO_2 and NADH, further increasing the supply of precursors necessary for succinate synthesis. This surplus of CO_2, together with that provided by $NaHCO_3$ (added to the culture medium), could be funneled to the pyruvate-oxaloacetate node by means of the over-expressed Ppc^{Ec}, which fixes carbon dioxide to pyruvate forming oxaloacetate, that, in turn, could be converted to succinate (Fig. 3).

To test this hypothesis, strains K1060 and DC1060, co-transformed with plasmids pSBF2 and pEcPpc, were grown for 48h in low aeration with the addition of 100 mM $NaHCO_3$. Two different concentrations of IPTG (0.1 mM and 1.0 mM) were used to get a better estimation of the relative weight of the conversion catalyzed by Ppc^{Ec} and $FDH1^{Cb}$ on succinate production. With the lowest concentration of IPTG, the *creC* mutant produced 2.7-fold more succinate than the parental strain (Fig. 4A). The yield of succinate on glucose followed this trend, being 0.04 and 0.12 mol mol^{-1} for K1060 and DC1060, respectively (Fig. 4B). When IPTG was supplied at 1.0 mM, succinate production was triggered, with marked increases in both titer and yield in both strains. The succinate yield of the Δ*creC* mutant strain over-expressing ppc^{Ec} and $FDH1^{Cb}$ was around 40% higher than that of the wild type (K1060/pSBF2-pEcPpc) in the same culture conditions.

The production of other metabolites in the strains carrying the plasmids (Fig. S2 in the Supplemental material) showed some modifications when compared to the strains without

390 plasmids (Table 2). For example, formate synthesis was lower in the strains containing plasmids
391 than in the plasmid-less counterparts, in accordance with the results expected from *FDH1*^{Cb}
392 overexpression.
393
394 In order to eliminate side products and further increase reducing power availability to stimulate
395 succinate production, additional modifications in the genetic background of strain DC1060 were
396 tested. The ethanol pathway was deleted to increase NADH availability, and *ackA* was also
397 eliminated to save carbon atoms in the form of acetyl-CoA, a substrate for succinate formation
398 via the glyoxylate pathway (46). The double mutant CE1060 (Δ*creC* Δ*adhE*) and the triple
399 mutant CEA1060 (Δ*creC* Δ*adhE* Δ*ackA*) were co-transformed with plasmids pEcPpc and pSBF2,
400 and succinate was measured in cultures of these strains grown in the same conditions described
401 above. In contrast to what was expected, these strains did not present much higher amounts or
402 yields of succinate when compared to *E. coli* DC1060/pSBF2-pEcPpc (Fig. 4). In all cases, a
403 sharp increase in succinate production was observed with higher amounts of IPTG. These results
404 indicate that over-expression of *ppc*^{Ec} and *FDH1*^{Cb} had a marked effect on succinate production,
405 while the mutations in *adhE* and *ackA* did not.
406
407 Acetate, formate, lactate, and pyruvate were measured in the cultures of the different strains (Fig.
408 S2 in the Supplemental data). Acetate formation in the *ackA* mutant was reduced compared to the
409 parental strain but not completely abolished, probably due to the activity of alternative pathways
410 for acetate formation (e.g., PoxB). In all cases, higher expression of the heterologous enzymes
411 (1.0 mM IPTG) tended to reduce the differences in the metabolic profile among the mutants, and
412 caused a decrease in the amounts of formate when compared to the low induction level (0.1 mM
413 IPTG).
414

Discussion

While analyzing different *arcA* mutants, a constitutive *creC* allele was discovered to be responsible for the peculiar phenotypic traits of one of the mutants that grew better than the others (18). In that study, the constitutive *creC510* allele was observed to ameliorate some of the phenotypic characteristics of Δ*arcA* mutants, and the effect was attributed to increased substrate consumption. Further work analyzed the carbon fluxes of strains harboring *arcA* and *creB* mutations in microaerobic glucose-limited chemostat cultures (47), revealing that these regulators share the control of carbon catabolism in these conditions. These results opened questions concerning the contribution of the CreBC system to central carbon metabolism under different oxygen availability conditions when the carbon source does not limit bacterial growth. This question was of special interest, since it was reported that CreC does not respond only to the composition of the media (i.e., whether it is rich or mineral medium) as it was initially believed (7), but its activation is also dependent on the aeration level and the carbon source (6).

To further analyze the metabolic effects of CreBC, three different aeration levels (high, low, and no aeration) were tested to determine the behavior of *creC* mutants considering different aspects of cell metabolism, such as the metabolite profile, enzyme activities, and redox state. These experiments showed that CreC has a clear effect over carbon distribution that varies in different oxygen availability conditions. CreC was observed to affect the accumulation of formate, lactate, acetate, ethanol and succinate. These effects were shown to be mediated by CreB, ruling out the possibility that they could be due to interaction of CreC with other non-cognate response regulators. This observation is not trivial, since CreC was originally associated with the *pho* regulon (48) interacting with the response regulator PhoB. This affirmation does not exclude, however, the possibility that other regulators could affect the expression of genes in common.

Differences in the distribution of metabolites in the aeration conditions analyzed in this work were also associated to the intracellular redox state. In previous studies that analyzed Δ*arcA* *creC510* double mutants, the constitutive *creC510* allele was observed to contribute to a high

444 ethanol/acetate ratio (18), while the opposite was observed in *creB* mutants (47). These
445 differences were also observed in this study. For example, ethanol accumulation, that normally
446 correlates with a reduced intracellular state (i.e., increased NADH/NAD^+ ratio), was lower in the
447 Δ*cre* mutants, and higher in the mutant carrying the constitutive *creC510* allele when aeration
448 was suppressed as compared to the parental strain.
449
450 The effects of CreC on the redox state of the cells were further analyzed by measuring the ratios
451 between reduced and oxidized cofactors. In all oxygen conditions, the strain carrying *creC510*
452 had a higher NADH/NAD^+ ratio than the wild-type strain and the Δ*creC* mutant, showing that
453 CreC contributes to a more reduced intracellular redox state. While the null mutant had
454 significant ($P < 0.05$) differences in NADH/NAD^+ ratios with both the parental strain and the
455 *creC510* mutant in low aeration, this was not observed in high aeration or no aeration, indicating
456 that the effect of CreC on redox potential was stronger in intermediate oxygen availability
457 conditions.
458
459 The determination of reduced and oxidized cofactors in the three aeration conditions tested
460 revealed additional interesting data. While in high aeration the NADPH/$NADP^+$ ratios were
461 higher than the NADH/NAD^+ ratios for all strains, the opposite was observed in un-aerated
462 cultures. This observation could have two different implications. The first is that the degree of
463 oxidation of the two cofactors in high aeration and no aeration reflects differences in cofactor
464 usage. NADP(H) is considered the preferred cofactor in anabolism and stress resistance
465 mechanisms, whereas NAD(H) is mainly related to catabolism and fermentation processes (49).
466 Therefore, the lower NADPH/$NADP^+$ ratio compared to the NADH/NAD^+ ratio in non-aerated
467 cultures is possibly related to a decrease in the NADPH content, which in turn is reflected in the
468 low biomass formation under these conditions. Another possible explanation could be that cells
469 regulate NADH/NAD^+ ratios more tightly than NADPH/$NADP^+$ ratios, as the latter were
470 observed to vary on a much wider range with respect to oxygen availability. In this scenario, the
471 ratio between reduced and oxidized NAD(H) is maintained with modest variations through

different oxygen availability conditions, while the pool of NADP(H) is mostly reduced in highly aerated cultures and mostly oxidized in non-aerated ones.

In this work, no significant variations were detected when the NADPH/$NADP^+$ ratios obtained for the different strains were compared within each condition, while differences in the NADH/NAD^+ ratios were observed in all aeration conditions. The oxidation state of NAD(H) has previously been reported to vary greatly in different genetic backgrounds (49-52), such as in redox regulatory mutants (53-55), so the differences in NADH/NAD^+ ratios between the strains carrying the *cre* variants support the hypothesis that CreC affects the intracellular redox state. Additionally, the results obtained in this work could reflect that the content of $NADP^+$ responds more strongly to variations in oxygen availability than to different genetic or metabolic backgrounds.

At least 120 proteins are shown to change their expression in response to a shift from aerobic to anaerobic conditions (2). The expression of over 30 operons (more than 70 genes) are under control of the Fnr regulator (56) and at least 40 operons are regulated by the two-component regulatory ArcAB system (57, 58), including 16 genes that encode proteins playing a role in carbon metabolism (16, 59). The ArcAB and Fnr global regulation systems are major controlling factors of gene expression, and in most cases they operate coordinately to fine-tune catabolism in response to oxygen availability (60). The metabolic changes observed in this work suggest that CreBC might also contribute to the regulation of the intracellular redox state, although it is likely that its role does not involve direct sensing of the redox state of the cells, but rather, as it has been suggested in previous work, a metabolic signal, such as a carbon catabolism intermediary (6).

The CreBC system was first reported to respond to growth in minimal media (7), and later shown to be active when cells are grown in gluconeogenic C sources or fermenting glucose (6). The results presented in this study, in which all experiments were performed using glucose as the C source, clearly indicate that the regulation mediated by CreBC is affected by oxygen availability.

In a mineral culture medium with excess glucose, high aeration did not offer a propitious environment for CreC activation, as shown by similar metabolic profiles (characterized by high acetate formation) and enzyme activities among the three strains. Mutations in CreC gave more conspicuous phenotypes when low or no aeration was supplied. This result is in accordance with the observations by Cariss et al. (6), that indicated that CreC was activated when cells were cultured in sealed flasks, limiting the oxygen supply. However, there was not a clear trend across the different aeration levels. The *in vitro* enzyme activity levels as well as the formation of some metabolites were not affected in the same manner by the absence of CreC at different aeration levels. Except for succinate, that had higher yields in the absence of CreC in both low or no aeration, the relative values of other organic acids differed in the two conditions. For example, in low aeration the null mutant accumulated more formate and acetate, but less lactate than the parental strain, while in the absence of aeration the opposite relationship was observed for acetate and lactate, with similar amounts of formate.

In an attempt to further characterize this effect, the activities of AckA and LdhA were determined, and both were observed to be affected by CreC. While *ackA* was previously shown to be under transcriptional regulation exerted by CreBC (7), *ldhA* was not thought to be affected by this regulatory system. This gene has also been observed to be negatively regulated by Mlc (which affects the expression of *pts* genes) (61), CsrA (a regulator of carbohydrate metabolism that influences glycogen biosynthesis, gluconeogenesis, glycolysis, and glycogen degradation) (62, 63), and positively regulated by ArcAB and CsrB (which antagonizes CsrA) (64). The differences in lactate production, together with those observed in LdhA activity, suggest that CreC could also have a regulatory effect over *ldhA*, in spite of the lack of a consensus *cre* tag in the region upstream from this gene. Further experiments need to be performed to assess whether these differences are directly mediated by CreBC regulation at the transcriptional level. For all cultures grown in low aeration, a correlation between enzyme activities and metabolite levels was observed, as higher acetate and lower lactate values in the Δ*creC* mutant compared to the parental strain corresponded to higher AckA and lower LdhA activities, but this correlation was not observed in non-aerated cultures.

Although enzymatic studies are typically performed in exponential cultures, enzyme activity was also determined in early stationary non-aerated cultures to investigate whether there was a correlation between *in vitro* enzyme activities in this growth phase and metabolite levels (measured in 24 h cultures). When analyzing this point of the growth phase, lactate production in strains K1060 and DC1060 correlated with LdhA activity, as a higher enzymatic activity was observed for *E. coli* DC1060, that accumulated more lactate in non-aerated exponential cultures. The conditions in which *ldhA* is normally expressed are anaerobiosis and acidic pH (33). Given that DC1060 grew more than K1060 and that cultures of DC1060 had a marked reduction in pH, it is possible that in the stationary phase the effects of the lower pH contributed to the higher LdhA activity observed. This, in turn, could result in higher lactate accumulation. In the exponential phase of non-aerated cultures, which had only slight differences in pH, both LdhA activity and lactate formation were lower for the null mutant, as observed in the cultures grown with high and low aeration. The only condition in which the Δ*creC* mutant had higher LdhA activity and produced more lactate than the parental strain was when these cultures had a much lower pH, so it is possible that this parameter affected LdhA activity, leading to increased lactate levels. In all other conditions, in which no differences in culture pH were observed between the strains, the Δ*creC* mutant produced less lactate and had lower LdhA activity, so it can be proposed that CreC has a positive regulatory effect on *ldhA*, the nature of which remains to be elucidated.

Concerning acetate, while the DC1060 mutant produced higher amounts of this metabolite than the wild-type strain in low aeration but not when aeration was suppressed, the *in vitro* AckA activity was higher for the mutant strain in both conditions, indicating that there was not a direct correlation between AckA activity and acetate accumulation. This suggests there must be additional factors that affect acetate levels in the cultures, which could be due to postranscriptional, allosteric, or even metabolic regulation. It cannot be ruled out that other enzymes might contribute to the effect observed, such as pyruvate oxidase (PoxB), that has been reported to be the main pathway for acetate production in stationary phase (30), although this enzyme is supposedly repressed in anaerobiosis (65).

559 In previous studies, it was seen that *ackA* is transcriptionally activated by CreC (7), and that in
560 the absence of CreB there is a reduced acetate flux when the cells are grown in continuous
561 cultures under carbon-limited conditions and restricted oxygen supply (47). However,
562 differences in aeration conditions have been shown to have dramatic effects over CreC
563 regulation (6). In our work, performed in the presence of an excess carbon source, the *creC* null
564 mutants displayed higher AckA activities compared to the wild-type strain, while the absence of
565 CreC had different effects over acetate accumulation according to the aeration level (low or no
566 aeration). Furthermore, the relative weight of the effects of Cre on other physiological
567 parameters analyzed in this study also depended highly on oxygen availability. While the
568 strongest effects on redox levels were observed in low aeration, a notable influence was seen
569 over growth in non-aerated cultures, and differences in metabolite distribution when compared to
570 the other strains also varied appreciably in the conditions analyzed. These results suggest that the
571 metabolic effects of the *cre* variants are highly susceptible to changes in culture conditions,
572 including oxygen availability.
573
574 The Δ*creC* mutation proved useful to increase the production of succinate. This compound is an
575 intermediary metabolite in the tricarboxylic acid cycle, and, under anaerobic conditions, can be
576 produced via the reductive arm of the cycle and the glyoxylate shunt, although the latter route
577 contributes less than the former. As NADH is required for its formation, different strategies have
578 been used to increase cofactor availability and to improve succinate production (66). It has been
579 proposed that the manipulation of the CreBC system (as well as ArcAB) could provide a relevant
580 tool for the modulation of central metabolism and reducing power availability aimed towards
581 biotechnological purposes (16), such as succinate production. Although the positive effect of the
582 *creC* deletion on succinate formation may not seem obvious from the point of view of the redox
583 balance, the increase in succinate production in *E. coli* DC1060 compared to the parental strain
584 was observed in all aeration conditions, and also when plasmids over-expressing
585 decarboxylating enzymes were added to both strains. The complementary strategy of enhancing
586 CO_2 fixation (through Ppc^{Ec}) and increasing the pool of NADH (through $FDH1^{Cb}$) at the expense
587 of excess formate worked satisfactorily. Additional mutations in structural genes did not produce

588 significant increases in the titer of succinate, even though they affected the secretion of other
589 acids. In general, the differences in succinate production between the mutants became less
590 relevant when other modifications were introduced, such as the expression of $FDH1^{Cb}$ and Ppc^{Ec},
591 indicating that the relative weight of the mutations affecting the different metabolic steps was
592 lower than the over-expression of these two heterologous genes. However, the *creC* mutants
593 continued to accumulate more succinate than the strain carrying the wild-type allele even when
594 the enzymes were over-produced in all strains, evidencing the role of the *creC* mutation in
595 boosting succinate synthesis. A hitherto unknown regulation exerted by CreBC on the enzymes
596 involved in the synthesis and/or consumption of succinate in *E. coli*, as observed in this work for
597 LdhA, cannot be ruled out. The relatively simple strategy of modifying the global regulation
598 exerted by CreBC offers an appealing alternative to the traditional, gene-by-gene metabolic
599 engineering strategies for the production of succinate (40, 46, 67, 68).
600
601 In conclusion, this work shows that CreC affects both carbon catabolism and the intracellular
602 redox state. The *creC* mutants were shown to exhibit different behaviors according to the degree
603 of oxygen limitation when grown in a mineral medium, with diverse effects over growth,
604 metabolites secretion, and redox balance. These results also reveal that, like other global
605 regulators, Cre influences many different aspects of bacterial physiology, although more research
606 is needed to elucidate the mechanisms by which CreC exerts its regulation. The diverse
607 metabolic effects of this regulator over the central biochemical network of *E. coli* make it a good
608 candidate for genetic manipulations to improve the formation of compounds of commercial
609 interest, such as succinate.
610
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612
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856 # FIGURE LEGENDS

857

858 **FIG. 1.** Determination of the NADH/NAD^+ and NADPH/$NADP^+$ ratios in *E. coli* K1060 (wild-
859 type strain), K1060C (carrying the constitutive *creC510* allele), and DC1060 (Δ*creC*). Cells were
860 grown in M9 minimal medium containing 30 g $liter^{-1}$ glucose under high aeration (A), low
861 aeration (B), and no aeration (C). Cells were harvested at mid-exponential phase. Results
862 represent the average ± standard deviation from duplicated measurements from at least two
863 independent cultures.

864

865 **FIG. 2.** Specific (Sp) acetate kinase (A) and lactate dehydrogenase (B) *in vitro* activities (act) of
866 cells grown in M9 minimal medium containing 30 g $liter^{-1}$ glucose under high aeration, low
867 aeration, and no aeration. The samples were harvested at mid-exponential phase, except under
868 anaerobic conditions, in which the activity was measured both in exponential (E) and in the early
869 stationary phase (S) (see Fig. S1 in the Supplemental material for detailed information on
870 sampling times). Results represent the average ± standard deviation from duplicated
871 measurements from at least two independent cultures.

872

873 **FIG. 3.** Diagram of the main metabolic pathways leading to succinate formation from glucose in
874 *E. coli*. The genes encoding Ppc^{Ec} (phosphoenolpyruvate carboxylase) from *E. coli* and $FDH1^{Cb}$
875 (NADH-forming formate dehydrogenase) from *Candida boidinii* were over-expresed in plasmids
876 pEcPpc and pSBF2, respectively (the corresponding reactions are highlighted in green). The
877 genes encoding AdhE (alcohol dehydrogenase) and AckA (acetate kinase) were knocked-out in
878 an attempt to enhance succinate accumulation (indicated by slanted red arrowheads). Note that
879 several reactions within the biochemical network were grouped for the sake of simplicity. EMP
880 pathway, Embden-Meyerhof-Parnas pathway; PEP, phosphoenolpyruvate; Acetyl-CoA, acetyl-
881 coenzyme A.

882

883 **FIG. 4.** Profile of succinate formation in the *E. coli* strains under study. Cells were grown in M9
884 minimal medium containing 30 g $liter^{-1}$glucose and 100 mM $NaHCO_3$ under low aeration for

48h. The *E. coli* strains tested were K1060 (wild-type strain), DC1060 (Δ*creC*), CE1060 (Δ*creC* Δ*adhE*), and CEA1060 (Δ*creC* Δ*adhE* Δ*ackA*). All bacteria were transformed with plasmids pSBF2 (carrying $FDH1^{Cb}$, a NADH-forming formate dehydrogenase from *Candida boidinii*) and pEcPpc (carrying ppc^{Ec}, the endogenous phosphoenolpyruvate carboxylase from *E. coli*). The expression of the genes in these plasmids was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) at two concentrations (0.1 mM and 1 mM). Succinate was assayed in culture supernatants and the results are reported as final concentration (concn.) (A) and yield of succinate on glucose ($Y_{Succ/Glc}$) (B). Results represent the average ± standard deviation from duplicated measurements from at least two independent cultures.

TABLES

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strain, plasmid or oligonucleotide	Relevant characteristics[a]	Reference or source
E. coli strains		
K1060[b]	F$^-$ *fadE62 lacI60 tyrT58*(AS) *fabB5 mel-1*	(69)
K1060C	Same as K1060, but *creC510* by K1060×P1(58-161,F-,SR)	This work
DB1060	Same as K1060, but Δ*creB* by allelic replacement	This work
DC1060	Same as K1060, but Δ*creC* by allelic replacement	This work
DBC1060	Same as K1060, but Δ*creB* Δ*creC* by allelic replacement	This work
CE1060	Same as DC1060, but Δ*adhE* by allelic replacement	This work
CEA1060	Same as CE1060, but Δ*ackA* by allelic replacement	This work
58-161,F-,SR[b]	F$^-$ *relA1 rpsL100*(Str^R) *spoT1 metB1 creC510*	(70)
58KF	Same as 58-161,F-,SR but Km^R by insertion of *kan* in an intergenic region between *yjjX* and *trpR*	This work
Plasmids		
pCP20	Helper plasmid used for *kan* excision; *Saccharomyces cerevisiae FLP* λ *c*I857 λPR *repA*(Ts), Ap^R Cm^R	(25)
pKD4	Template plasmid carrying the FRT-*kan*-FRT cassette, Km^R	(24)
pKD46	Helper plasmid expressing the λ-Red functions, Ap^R	(24)
pSBF2	Plasmid pDHK30 (71) carrying *FDH1* from *Candida boidinii* under control of the *lac* promoter, Km^R	(43)
pEcPpc	Plasmid pTrc99A (72) carrying *ppc* from *E. coli* under control of the *lac* promoter, Ap^R	(39)
Oligonucleotides[c] (5' → 3')		
cre/F	TAG GCC TGA TAA GAC GTG GCG CAT CAG GCA	This work

	TCG TGC ACC GAA TGC CGG ATG TGT AGG CTG GAG CTG CTT C *(K1060C construction)*	
cre/R	GCC GCG TCT TAT CAT GCC TAC CAA ACA TAT TGA AAT TAC GGG TAT TTG TAC ATA TGA ATA TCC TCC TTA G *(K1060C construction)*	This work
creB-KF/F	TTA GCG CGG TTC CTG TCA TGC CGT GGC GGC AAT AAC AGA GGC GAT TTA TGG TGT AGG CTG GAG CTG CTT C *(DB1060 and DBC1060 construction)*	This work
creB-KF/R	GCC CAG CAA CAA CCG CAT GCC GAT ACG CAT TAC AGG CCC CTC AGG CTA TAC ATA TGA ATA TCC TCC TTA *(DB1060 construction)*	This work
creC-KF/F	GTC AAA GAA GTT AAA CCG GGC GTG CGA AGA GCA ACG GAG GGG ACG TTG ATC GTG TAG GCT GGA GCT GCT TC *(DC1060 construction)*	This work
creC-KF/R	GAC GTG TTC CTG ATC CAC TTC GGC GCT TAG CGT GAT GCA ACC GCT CTC GGG CAT ATG AAT ATC CTC CTT AG *(DC1060 and DBC1060 construction)*	This work
DW-*ack*/F	AAC TCA GCG GGA CAA CG *(CEA1060 construction)*	This work
DW-*ack*/R	GAA AGC AGA CCT TCA ACG *(CEA1060 construction)*	This work
DW-*adh*/F	AGA CGC GCT GAC AAT ACG *(CE1060 and CEA1060 construction)*	This work
DW-*adh*/R	GCC ACC AGA CGC ATA ACC *(CE1060 and CEA1060 construction)*	This work

[a] Antibiotic markers: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; and Str, streptomycin.

[b] Strain obtained through the *E. coli* Genetic Stock Center, University of Yale, CT.

[c] The use of each oligonucleotide in the construction of mutant strains is indicated in parentheses.

906 **Table 2.** Growth and fermentation profile of the strains under study under different conditions of
907 oxygen availability.

Aeration condition	*E. coli* strain	Biomass (g_{CDW} $liter^{-1}$)	Yield of fermentation metabolites on biomass (mmol g_{CDW}^{-1})				
			Succinate	Lactate	Formate	Acetate	Ethanol
High aeration	K1060	1.29 ± 0.02	0.4 ± 0.1	1.0 ± 0.1	0	14 ±2	9.0 ± 1.0
	K1060C	1.25 ± 0.01	1.0 ± 1.0	1.0 ± 0.1	0	12 ±1	9.0 ± 0.1
	DC1060	1.25 ± 0.01	0.3 ± 0.1	1.0 ± 0.1	0	11 ±1	6.0 ± 0.1
Low aeration	K1060	0.38 ± 0.02	0.5 ± 0.2	46 ± 8	17 ± 3	89 ± 13	18 ± 5
	K1060C	0.39 ± 0.02	0.4 ± 0.1	48 ± 7	22 ± 3	91 ± 9	17 ± 2
	DC1060	0.36 ± 0.01	6.9 ± 0.3	22 ± 2	44 ± 3	127 ± 10	19 ± 3
	DB1060	0.39 ± 0.01	6.3 ± 0.2	19 ± 1	48 ± 1	120 ± 4	N.D.
	DBC1060	0.36 ± 0.01	6.7 ± 0.3	20 ± 1	50 ± 5	125 ± 9	N.D.
No aeration	K1060	0.14 ± 0.01	0.2 ± 0.1	27 ± 8	61 ± 2	118 ± 4	68 ± 10
	K1060C	0.11 ± 0.04	0.1 ± 0.1	19 ± 8	41 ± 11	150 ± 7	120 ± 23
	DC1060	0.26 ± 0.01	9.4 ± 0.9	45 ± 5	58 ± 5	46 ± 9	29 ± 12
	DB1060	0.23 ± 0.02	11.0 ± 1.0	46 ± 4	56 ± 4	53 ± 10	N.D.
	DBC1060	0.24 ± 0.02	11.6 ± 0.2	51 ± 6	61 ± 5	55 ± 3	N.D.

908

909 Cells were grown in M9 minimal medium supplemented with 30 g $liter^{-1}$ glucose for 24 h. CDW,
910 cell dry weight; N.D., not determined. Results represent the mean value ± standard deviation of
911 three determinations from at least four independent cultures. The *E. coli* strains used are K1060
912 (wild type), K1060C (*creC510,* constitutive *creC* allele), DC1060 (Δ*creC*), DB1060 (Δ*creB*),
913 and DBC160 (Δ*creB* Δ*creC*). Results represent the average ± standard deviation from triplicate
914 measurements from at least three independent cultures.